Luna Prenatal Test White Paper

A cell-based prenatal genetic test

This document is prepared related to the launch of a new prenatal test.
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Introduction

Currently, the most widely used laboratory procedures for prenatal genetic testing are amniocentesis, chorionic villus sampling (CVS), and cell-free noninvasive prenatal testing (cfNIPT). CVS and amniocentesis are considered diagnostic but are invasive and carry a small risk of pregnancy loss. In contrast, cfNIPT is a risk-free screening test, but results are not diagnostic. It is a high priority for patients and providers to have available a prenatal genetic test that is both diagnostic and risk-free. The Luna Prenatal Test is a step in this direction, but it is not considered fully diagnostic for aneuploidy due to the possible occurrence of placental mosaicism. The potential to use fetal cells in maternal blood to derive noninvasive prenatal accurate results has been known since 1969,[1] but various technological challenges have hindered the introduction of such a test into the clinical arena. The Luna Prenatal Test (a cell-based prenatal genetic test) as described here overcomes these challenges and meets the need for a safe and reliable prenatal test that is the most accurate and most informative noninvasive test to date. The Luna Prenatal Test is noninvasive, risk-free, highly accurate, and available early in pregnancy.

Concepts for the Luna Prenatal Test

The development of cfNIPT has changed attitudes regarding risks for prenatal diagnosis. Although the risks from CVS and amniocentesis are small, the availability of risk-free testing has led many women to resist invasive testing, and the number of CVS and amniocentesis procedures has decreased substantially since the access to cfNIPT.[2] With the long-standing awareness of fetal cells in the maternal circulation, academic investigators and biotechnology companies have pursued the use of fetal cells for prenatal diagnosis intensely for decades. One example of a high-profile effort published in 2002 (20 years ago) was an NIH funded “prospective, multicenter clinical project to develop non-invasive methods of prenatal diagnosis”.[3] This trial, designated NIFTY I, concluded that “technological advances are needed before fetal cell analysis has clinical application.” One key advance over the last decade is the ability to perform molecular analysis (DNA and RNA sequencing) on single cells.[4] Multiple publications over the last five years have demonstrated the feasibility of using fetal trophoblasts in the maternal circulation to detect chromosomal abnormalities in the fetus.[5-8] Direct analysis of fetal trophoblasts from maternal blood showed that many single cells yielded good copy number data using next generation sequencing (NGS), but some cells were apoptotic (dying), and the DNA was poor quality and useless for diagnosis, while other single cells were in the midst of replication of their DNA (in S phase of the cell cycle) and could not be used to detect small deletions or duplications in the genomic DNA.[7] These considerations favor analyzing one cell at a time.

Another important benefit underlying the Luna Prenatal Test is that single fetal cells provide access to 100% pure fetal DNA, while cfNIPT analyzes a mixture of fetal placental and maternal DNA

where fetal fraction defines the proportion of the DNA that is fetal (usually 2-20%).[10] In addition, there is no evidence that fetal trophoblasts can persist from one pregnancy to the next.

Technical workflow and validation overview

The rarity of most fetal chromosomal abnormalities necessitated a three-part approach to demonstrating the analytical performance of the Luna Prenatal Test (**Fig. 1**). Pregnant patients were recruited into the study in two groups; the first group (59 women) were high-risk and were undergoing concurrent diagnostic testing by either CVS or amniocentesis, while the second group was larger (158 women) and low-risk and had no known diagnostic testing. To ensure the validation set encompassed an appropriate range of whole chromosome aneuploidies and small deletions and duplications, an additional set of seven Coriell cell lines harboring known chromosome abnormalities made up the third set of test samples. For all three test groups, single cells enriched from maternal whole blood (or control blood in the case of cell lines), were processed individually to obtain pure fetal or cell line DNA. The DNA from each individual cell was sequenced by low-pass massively parallel sequencing technology to generate a genome-wide copy number profile, which was evaluated for genome-wide copy number changes larger than 1.5 Mb.

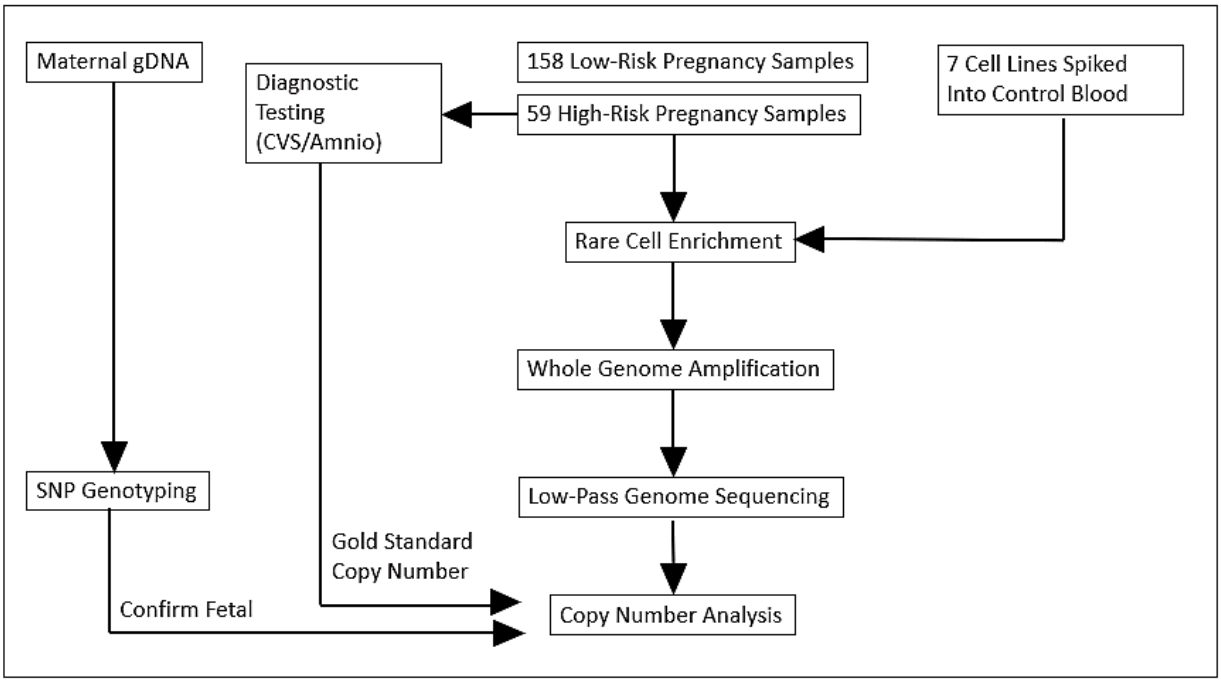


Figure 1. Flowchart showing the main steps of the validation process

Methodology Four 10 mL tubes of blood (Cell-Free DNA BCT®, Streck) were collected carefully to maximize free flow and avoid any clotting; blood was shipped overnight at ambient temperature on the day the blood was collected to the Luna Laboratory in Houston, TX. Blood was processed using a lysis procedure to eliminate red blood cells (RBC). The nucleated cell fraction was stained with antibodies to cytokeratin (CK). CK positive cells were processed with a microfluidic sorting step to enrich ~200 fold for CK-positive cells. The enriched cell fraction was loaded into CyteSlides (RareCyte, Seattle, WA) and subjected to automated cell scanning using a CyteFinder/CytePicker (RareCyte). Candidate cells were reviewed in a gallery, and presumptive fetal trophoblasts were picked manually using a micromanipulator within the CytePicker and delivered to microfuge tubes. Occasional cells were picked

as trophoblast doublets, on the assumption that they were adjacent cells in the placenta. Individual cells were subjected to whole genome amplification using the PicoPLEX® Single Cell WGA Kit (Takara), and a DNA library suitable for NGS was prepared using the Nextera XT DNA Library Preparation Kit (Illumina). DNA was sequenced to obtain 1-5 million reads, and the read data were analyzed using the NxClinical (NxC) software (BioDiscovery). Up to five cells were analyzed for each case of singleton fetus. The data were reviewed, and initial evidence of copy number gains or losses were flagged for final interpretation and sign-out by ABMGG certified lab directors.

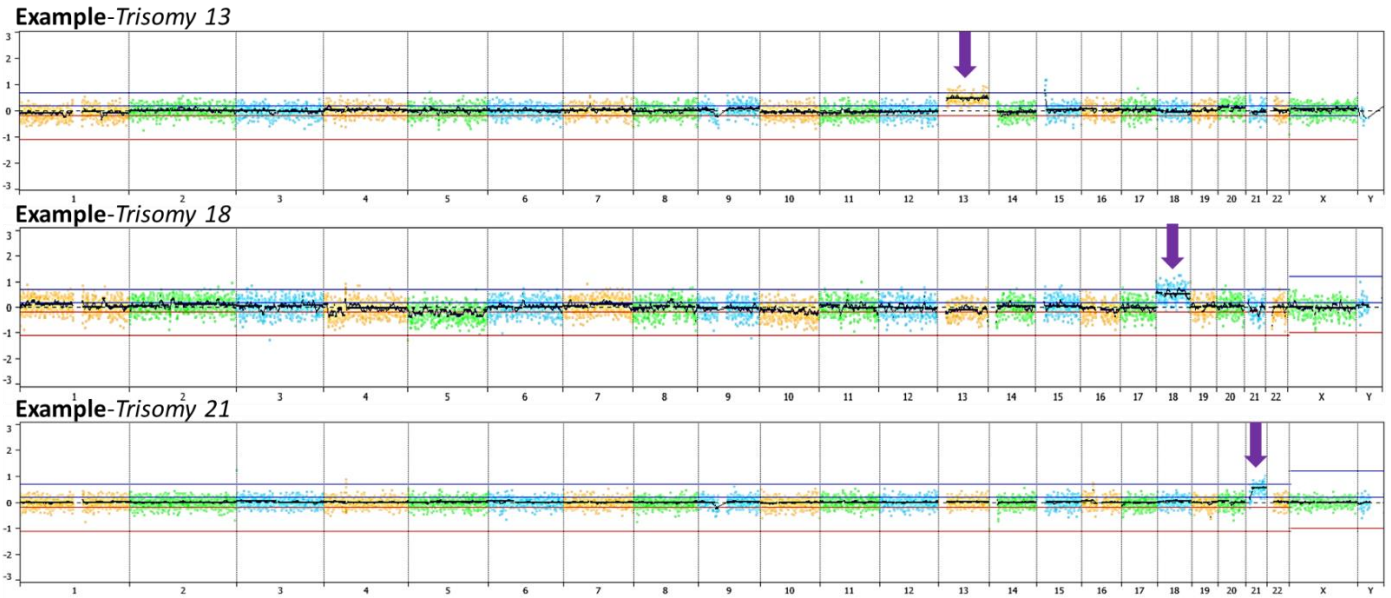
Fetal cell genotyping. Maternal genomic DNA was isolated concurrently from the blood sample and analyzed using a custom Illumina Global Screening Array v2 SNP array at an outside lab. The full SNP data for the mother were compared to limited SNP data for each cell from single cell NGS reads, and each cell was documented by genotyping to be of fetal and not maternal origin based on hundreds to thousands of SNP alleles present in the cell but not in the mother. Egg donation and surrogate maternal factors were considered.

Scoring Quality of NGS data. Importantly, every cell subjected to NGS analysis is given one of three scores for aneuploidy and deletion/duplication (del/dup) as below by a genomics specialist.

1. Scorable for aneuploidy + 1.5 Mb del /2.0 Mb dup resolution
2. Scorable for aneuploidy only
3. Unscorable, not used for further analysis

Unscorable (equivalent to unusable) cells were either apoptotic cells, instances where the cell was lost in processing, cells with very low mappable reads, or the data were otherwise unsuitable for analysis due to unknown reasons. Cells scorable for aneuploidy only resolution were often cells in S phase of the cell cycle where numerous small genomic segments not yet replicated cannot be distinguished from small deletions. Cells scorable for aneuploidy + 1.5 Mb del/2.0 Mb dup resolution had very high quality NGS data and very few putative gains or losses called by the NxC software.

Examples of results. A few examples of typical NGS plots from single cells are shown in **Fig. 2.**



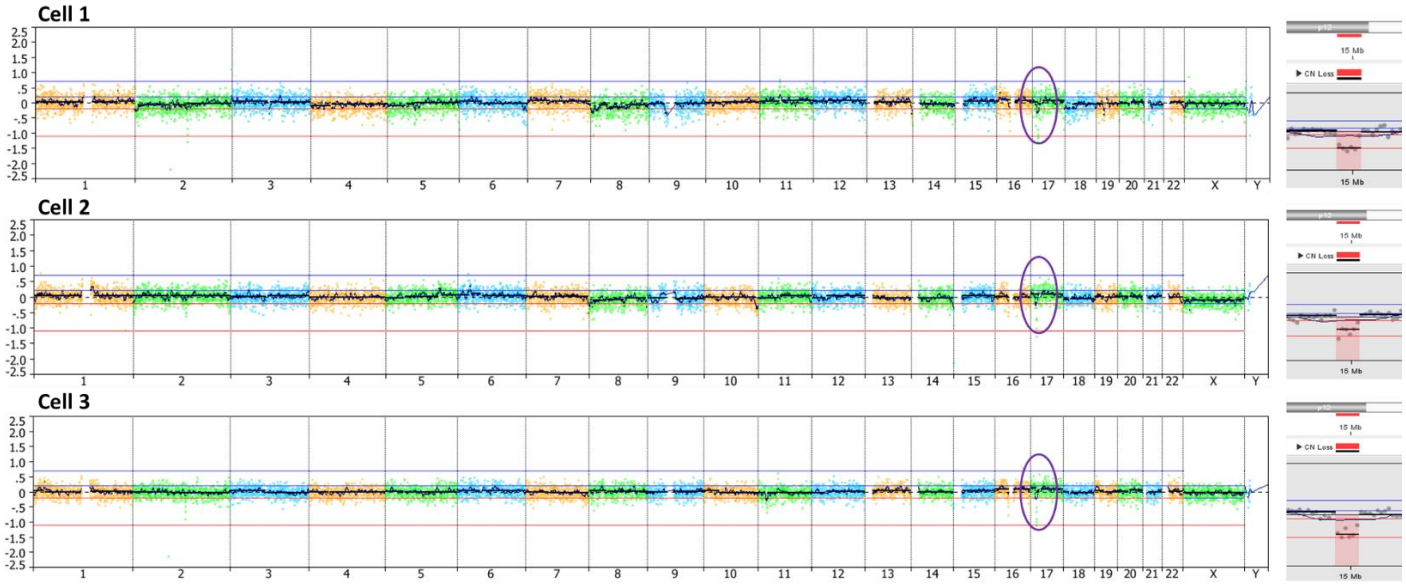


Figure 2. Examples of copy number analysis for single cells using the Luna Prenatal Test methodology. Genome wide copy number is plotted from chromosome 1 on the left to X and Y on the right in a format which is typical for display of chromosomal microarray data. For the upper section, each of three trophoblasts shown represent trisomy 13, trisomy 18, and trisomy 21 from top to bottom, respectively. The lower section shows examples of three single abnormal cells spiked into normal male blood with the cells recovered using tracking dye and the Luna methodology. A lymphoblast cell line with a 1.5 Mb deletion in the CMT1A region was used. In the genome-wide plot, there is evidence of a deletion on chromosome 17 (purple ovals). Zooming in to the relevant region on chromosome 17 on the far right demonstrates the 1.5 Mb deletion in each cell. All three cells shown were ranked as scorable for aneuploidy + 1.5 Mb del/2.0 Mb dup resolution.

Validation Studies

The validation studies for the Luna Prenatal Test were performed in three **Test Sets** (**Table 1**).

Table 1. Validation studies

Test Set	Sample number	Characteristics
1	59 (→ 200)	High risk. Comparison to CVS and amniocentesis
2	158	Low risk. From routine pregnancies
3	148 cells	Seven cell lines with aneuploidy or deletion

Test Set 1 (High risk) was designed to determine sensitivity and specificity when Luna Prenatal Test results were compared to CVS or amniocentesis results on the same cases. A total of 59 samples were received from three recruitment sites: Columbia University Irving Medical Center (Dr. Ronald Wapner), Icahn School of Medicine at Mount Sinai. (Dr. Joanne Stone) and Yale School of Medicine (Dr. Audrey Merriam). The characteristics of these samples are shown in **Table 2.** Any woman undergoing CVS or amniocentesis at these sites was eligible for participation, and the indications for performing testing were heterogeneous but many were high risk including cytogenetic risk, monogenic risk, and abnormal ultrasound. In all cases, blood was drawn prior to the invasive procedure. The results from CVS or amniocentesis served as accepted standard of care data for comparison to Luna Prenatal Test results. This comparison allows us to establish accuracy, specificity, sensitivity, positive predictive value (PPV), and

negative predictive value (NPV) of the Luna Prenatal Test. The analysis was restricted to aneuploidy since there were no instances of pathogenic deletion or duplication.

Results for Test Set 1. The protocol called for all clinical information provided to the CVS or amniocentesis lab including cfNIPT results to be provided to the Luna lab, but this did not occur in two cases with balanced translocation parents. Otherwise, the Luna lab was blinded to the CVS and amniocentesis results. It was not the intent to enroll triplet pregnancies, but one triplet pregnancy was enrolled. The specific findings of the triplet case are provided, but these data were not included in sensitivity, specificity, and other calculations.

Table 2. Subject and sample information associated with the Test Set 1 cases received.

Number of Samples (57 singleton and 2 twin)	59
Time in Transit (days)	1-5 days (average: 1.8 days ± 1.2 days)
Sample volume (mL)	20.8-38.3mL (average: 33.4mL ± 4.5 mL)
Gestational Age (weeks, days)	10 weeks 3 days-22 weeks 4 days (average: 14 weeks ± 3 weeks 5 days)
Maternal Age (years)	23-44 years old (average: 35.5 years old ± 4.7 years)
Recruitment Site	34 Columbia, 18 Mt. Sinai, 7 Yale

Figures 3 and **4** show the number of scorable cells obtained for the 59 samples received in Test Set 1. There were seven cases of aneuploidy (one T13, one T18, three T21, one T22, and one 47,XXX). The T13 pregnancy, two of the T21 pregnancies, and one 47,XXX pregnancy had positive cfNIPT testing for the abnormality identified. One of the samples was an opposite sex twin pregnancy with Luna Prenatal Test data for both twins, and therefore, two fetuses were available for comparison. Many of the normal cases were scored only for aneuploidy only resolution on the Luna Prenatal Test as shown in Figure 4.

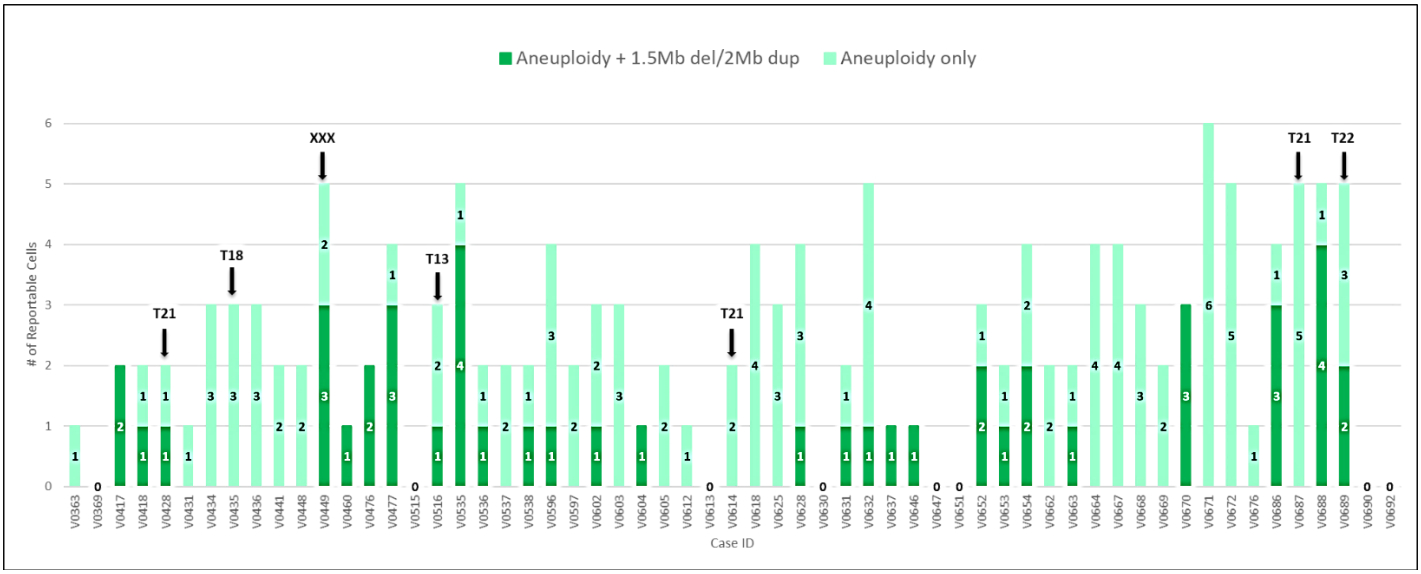


Figure 3. **Results of single trophoblast copy number analysis for 59 cases undergoing CVS or amniocentesis under Test Set 1**. Cell data are provided for each sample received by the Luna lab. Each rectangle represents a cell with dark green for aneuploidy + del/dup at 1.5/2.0 Mb resolution and light green for aneuploidy only resolution.

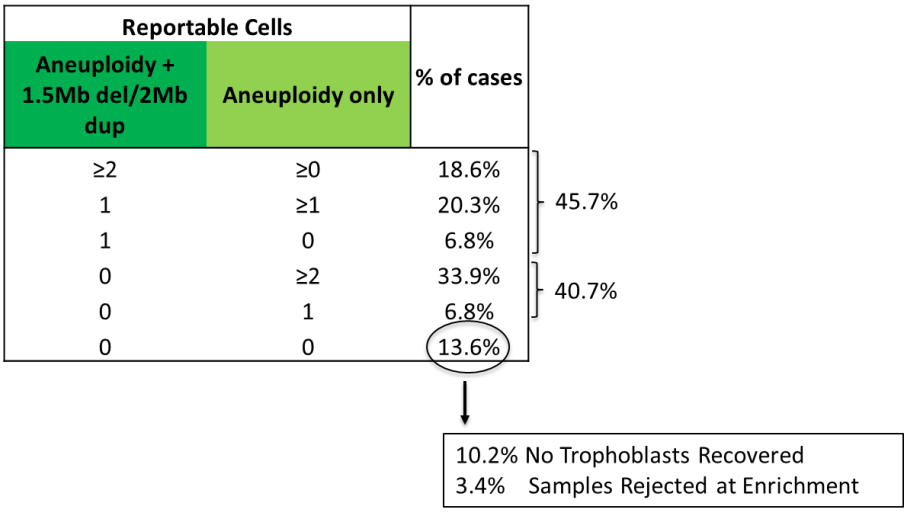


Figure 4. Tabulation of scorable cell data for Test Set 1. There were 27 samples with at least one cell scorable for aneuploidy + 1.5 Mb del/2.0 Mb dup, 24 samples with at least one cell scorable for aneuploidy only and 8 samples with no scorable cells for the Luna Prenatal Test.

Exclusions. Of the 59 samples received, 11 samples were excluded from comparison with amniocentesis or CVS results and statistical analysis (accuracy, sensitivity, specificity, PPV, and NPV) due to the following reasons: 1) amniocentesis or CVS results were not available for 2 samples, 2) no amniocentesis or CVS results were available for one sample due to confidentiality reasons, 3) fetal demise occurred prior to planned CVS testing for one sample with T22 found in all 5 cells by the Luna Prenatal Test, 4) one triplet pregnancy, 5) 4 samples where no trophoblasts were recovered, and 6) 2 samples were rejected at the enrichment stage due to sample quality. **Table 3** below shows complete agreement on cytogenetic diagnoses for the 49 fetuses in 48 pregnancies. There were 41 fetuses including one opposite sex twin pair with normal cytogenetic results. There were two fetuses reported as normal by Luna where the cytogenetic result was balanced translocation which was interpreted as aneuploidy agreement. Despite the protocol indicating that the Luna lab should receive all data that would go to the CVS or amniocentesis lab, the Luna lab was not informed of the presence of a balanced translocation parent in each case.

Table 3. Comparison of CVS and amniocentesis with Luna Prenatal Test for aneuploidy.

Diagnostic result	Number of cases CVS/amnio	Number of cases Luna Prenatal Test
Normal 46,XX or 46,XY	39 normal	39 normal
Balanced translocation	2 bal trans	2 normal = bal trans
Normal opp. Sex twins	Normal,1 46,XX, 1 46,XY	Normal,1 46,XX, 1 46,XY
Trisomy 21	3 trisomy 21	3 trisomy 21
Trisomy 13	1 trisomy 13	1 trisomy 13
Trisomy 18	1 trisomy 18	1 trisomy 18
47,XXX	1 47,XXX	1 47,XXX

Triplet case omitted.
T22 case omitted.
Case with confidential result omitted.

Conclusions from Test Set 1.
For the 48 pregnancies (49 fetuses) where Luna Prenatal Test results were obtained and included in the statistical analysis, the accuracy is 100%, specificity 100%, sensitivity 100%, PPV 100%, and NPV 100% as shown in **Fig. 5**. While this is a small sample set, the plan is to extend **Test Set 1** to 200 samples. Currently, the confidence intervals are wide for accuracy, sensitivity, and specificity.

		Chorionic Villus Sampling (CVS) or Amniocentesis	
		Positive	Negative
Luna Prenatal Test	Positive	True Positive (6)	False Positive (0)
	Negative	False Negative (0)	True Negative (43)

Statistic	Value	95% Confidence Interval (CI)
Accuracy	100%	92.75% to 100.00%
Sensitivity	100%	54.07% to 100.00%
Specificity	100%	91.78% to 100.00%
Positive Predictive Value	100%	N/A
Negative Predictive Value	100%	N/A
Disease Prevalence	12.24%	4.63% to 24.77%

Figure 5. Luna Prenatal Test Validation Study-Statistical Analysis

Test Set 2 was designed to assess the success rate for recovering trophoblast cells and for achieving NGS analyses that could be scored for aneuploidy + 1.5 Mb del/2 Mb dup or for aneuploidy only. Blood was collected from healthy pregnant volunteers who were widely distributed across 35 states in the US. Participants were recruited through social media and consented by a genetic counselor employed by Luna. A phlebotomist visited the home, drew blood, and delivered the sample for shipping. Each sample was processed with the methods that are being used for the launch of the test. In **Test Set 2**, a first blood sample was collected from 158 women. About a third of the way through the study, an initiative to collect a redraw of a second blood sample was implemented for samples where 0 or 1 cells were recovered in the first sample. The characteristics of the samples in **Test Set 2** are provided in **Table 4.**

Table 4. Subject and sample information associated with the Test Set 2 cases

	Test Set 2-Original Draws only	Test Set 2-Redraws only
Number of Samples	158 original draws	26 Redraws
Time in Transit (days)	1-4 days (average: 1.2 day ± 0.7 days)	1-6 days (average: 1.3 days ± 1.1 days)
Sample volume (mL)	10.8-41.7 mL (average: 35.4 mL ± 4.4 mL)	24.4-41.7 mL (average: 38.4 mL ± 3.6 mL)
Gestational Age (weeks, days)	6 weeks 5 days-17 weeks 6 days (average: 12 weeks 3 days ± 2 weeks 3 days)	10 weeks-20 weeks 4 days (average: 16 weeks ± 3 weeks 1 day)
Maternal Age (years)	20-44 years old (average: 31.6 years old ± 4.4 years)	25-40 years old (average: 30.8 years old ± 3.9 years)
Recruitment Site	35 U.S. States	17 U.S. States

Results for Test Set 2. There were 158 first draw samples and 26 redraw samples from 158 pregnancies. The overall information for NGS scorable cells is shown in **Fig. 6.** Focusing on the combined data from first draw and selected redraws, one or more scorable cells were obtained for

91.1% of samples so that a report with results would be issued for 91.1% of cases, and 8.9% of cases with a final result of “No scorable cells” (**Fig. 7**). Under ideal circumstances, the success might be slightly higher because we did not request redraws in the first 40 cases, and not all women who were asked for a redraw gave a second blood sample. At least one cell scorable for aneuploidy + 1.5 Mb del/2.0 Mb dup resolution was obtained in 75.3% of cases. Abnormalities in the samples included two with T21, three with 45X (one mosaic), and one each with a deletion of chromosomes 3, 5, or 15 (Prader-Willi/Angelman). Two samples revealed mosaic trisomy 20.

It is of interest to compare success in recovering scorable cells as related to gestational age as shown in **Fig. 8**. From these data, we suggest that the optimal time to perform the test is 9-14 weeks of gestation, though it is also feasible to perform the test as early as 8 weeks or as late as 5-22 weeks. If cells are obtained, the test is reliable, but there is a slightly increased risk of not obtaining scorable cells outside of the 9-14-week range.

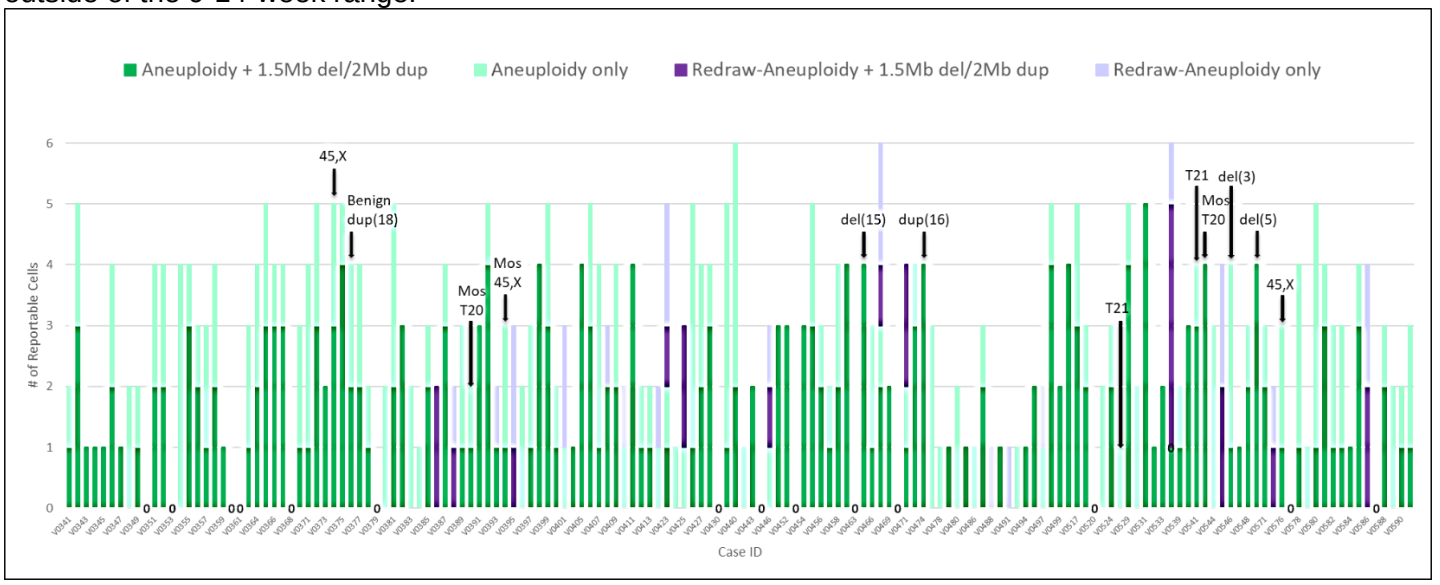


Figure 6. **Data for NGS scorable cells for 158 patients**. Each column represents a patient. Each rectangle represents a cell with dark green indicating aneuploidy + 1.5 Mb del/2.0 Mb dup resolution from first draw and light green indicating aneuploidy only resolution from first draw. Dark purple is for aneuploidy + 1.5 Mb del/2.0 Mb resolution from redraw and light purple for aneuploidy only resolution from redraw.

Figure 7. Tabulation of scorable cell data for Test Set 2. There were 119 samples with at least one cell scorable for aneuploidy + 1.5 Mb del/2.0 Mb dup, 25 samples with at least one cell scorable for aneuploidy only, and 14 samples with no scorable cells for the Luna Prenatal Test .

Original Draw + Redraw			
Reportable Cells			
Aneuploidy + 1.5Mb del/2Mb dup	Aneuploidy only	% of cases	
≥2	≥0	46.2%	
1	≥1	20.9%	75.3%
1	0	8.2%	
0	≥2	9.5%	15.8%
0	1	6.3%	
0	0	8.9%	

2.5% No Trophoblasts Recovered
6.4% No Reportable Cells

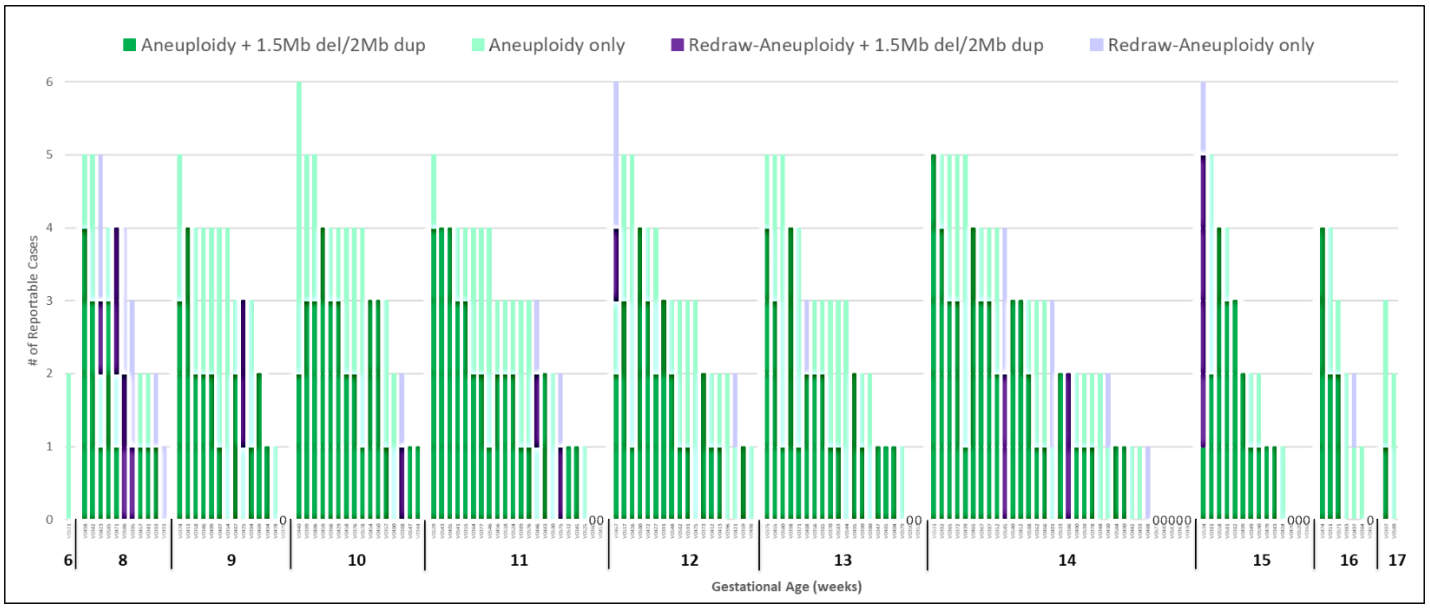


Figure 8. Data for NGS scorable cells for 158 patients in Test Set 2 sorted by gestation.

Table 5. Chromosomal abnormalities in 158 low risk pregnancies			
Result		# of cases	% of cases
Normal	46,XX or 46XY	132	83.5
Abnormal	45,X (1 mosaic)	3	1.9
	Trisomy 21	2	1.3
	del(3)(p26.3-p26.1) (3.9Mb)	1	0.6
	del(5)(p15.33) (3Mb)	1	0.6
	del(15)(q11.2 - q13.1) (6.3Mb)	1	0.6
	dup(16)(p13.11) (1.9Mb)	1	0.6
	Trisomy 20 (both mosaic)	2	1.3
Likely benign	dup(18)(p11.32) (2.2Mb)	1	0.6
No scorable cells	No results	14	8.8
	Total # of cases	158	

Concordance: Of the one hundred and fifty-eight (158) singleton pregnancy cases, one hundred and eighteen (118) cases had two or more reportable cells. Note, this count excludes three mosaic cases (two T20 and one 45,X) which by definition have cells of differing results. All cells (total 404 cells) within each of those 118 cases showed concordant results when assessing for clinically significant copy number abnormalities, meaning all cells within one case had the same findings, whether normal or abnormal (concordance = 100%). Thus, even a single cell has high predictive value if mosaicism is not present.

Test Set 3 utilized human cultured cells (lymphoblast or fibroblast obtained from Coriell Cell Repository) with known aneuploidy or deletion as shown in **Table 6**. Out of a total of 148 scorable cells, the known abnormality was detected and called by the NxC software in every cell except one. Thus, the analytic sensitivity and specificity were 100% with the expected finding being reported in every cell. This included inter-prep and intra-prep replicates as shown in **Table 7**.

Table 6. Cell line IDs and associated abnormalities

Coriell ID	Cell Line ID	Abnormality	Size
GM03102	C0001	47,XXY	whole chromosome
GM03538	C0002	Trisomy 18	whole chromosome
GM03716	C0003	Trisomy 21	whole chromosome
GM22049	C0004	del(15)(q11.1-q13.3)	12 Mb
GM23007	C0005	del(1)(p36.33-p36.22)	9.2 Mb
GM17942	C0006	del(22)(q11.21)	2.8 Mb
GM24084	C0007	del(17)(p12)	1.5 Mb

Table 7. Overview of Inter-prep and Intra-prep replicates

Interprep Replicates		
Replicate 1	**Replicate 2**	**Replicate 3**
C0001-1	C0001-2	C0001-3
C0002-1	C0002-2	C0002-3
C0003-1	C0003-2	C0003-3
C0004-1	C0004-2	C0004-3
C0005-1	C0005-2	C0005-3
C0006-1	C0006-2	C0006-3
C0007-1	C0007-2	C0007-3

Intraprep Replicates		
Replicate 1	**Replicate 2**	**Replicate 3**
C0002t-1	C0004t-1	C0007t-1
C0002t-2	C0004t-2	C0007t-2
C0002t-3	C0004t-3	C0007t-3

Clinical and Analytical Performance Assessment Summary. The clinical and analytical performance characteristics of the Luna Prenatal Test were evaluated for accuracy, precision (repeatability and reproducibility), and clinical and analytical sensitivity and specificity. Clinical concordance was verified between Luna results and reference laboratory-confirmed results as shown in **Table 3**. Overall, clinical sensitivity, specificity, PPV, and NPV were each 100% for all the observed conditions. Additionally, reproducibility and repeatability studies were performed using the same sample set to demonstrate precision of results across multiple set-ups of the assay (inter-run precision). Three replicates of a single sample on a single run were performed to demonstrate precision of test results under the same operating conditions (intra-run precision). The observed results from each of runs performed were 100% concordant. In addition, we performed a Limit of Detection Analysis by spiking in Coriell cell lines harboring characterized copy number abnormalities, which confirmed that our clinical Luna Prenatal Test pipeline can detect copy number losses down to 1.5 Mb (**Table 6**).

Potential for redraw and no result cases

Fetal trophoblasts were not recovered in every blood sample. Failure to recover cells is not an indication of any abnormality with the pregnancy and may occur more frequently in normal pregnancies as the number nucleated fetal cells is reported to be increased with some aneuploid fetuses.[11] In 26

cases with 0-1 cells recovered, a second blood sample (a redraw) was processed and resulted in additional scorable cells in 77% of cases.

What is the evidence that the Luna Prenatal Test is highly accurate?

The Luna Prenatal Test examines one fetal cell at a time, and an interpretation is made as to whether there is any pathogenic copy number change or not. There is no statistical estimate of the risk of an abnormality. Rather each cell is scored and assigned a genotype for the presence or absence of any abnormality. The data are very similar in format to those obtained when a chromosomal microarray is performed after CVS or amniocentesis. There is no interference from maternal genotype abnormalities. There is no statistical cutoff that is needed. **Test Set 2** of the validation study demonstrated complete intra-case concordance for 404 cells from 118 cases. **Test Set 1** of the initial validation study demonstrates complete agreement when the Luna Prenatal Test and CVS or amniocentesis are performed on the same singleton pregnancy and one opposite-sex twin pregnancy. We do not claim that the Luna Prenatal Test is diagnostic for aneuploidy, because there are recommendations from professional organizations that a final diagnosis should not rest on testing of trophoblasts alone. A publication by Van Opstal and Srebniak provides excellent guidance on the relative value of CVS (especially mesenchymal core analysis) and amniocentesis to confirm positive aneuploidy results based on trophoblast DNA.[12]

Interpretation

Each single cell is assessed for scorability for aneuploidy and del/dup at 1.5/2.0 Mb resolution and aneuploidy only resolution. Each cell is signed out as normal (no pathogenic copy number abnormalities detected) or as having a specific copy number abnormality. The final interpretation for the case is based on the cumulative findings in all the scorable cells. The number of cells scorable for aneuploidy + del/dup at 1.5/2.0Mb or aneuploidy only resolution is specified in the report.

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